EXHIBIT B

Novartis Pharma Research

Adenoviral vector secreting Endostatin: Av3mEndo

Therapeutic Area: GTI, Tumor Targeting Program

Characterization of Av3mEndo in Animal Tumor Models

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Introduction

Adenoviral vector encoding secreted murine endostatin, Av3mEndo, has been constructed and characterized (1). The detailed *in vitro* study has been described in the previous report "Construction and characterization of a recombinant adenoviral vector encoding secreted murine endostatin *in vitro*" (1). In this study, we focus on the *in vivo* efficacy study in various animal tumor models, naturally occurring colon liver metastasis model, B16F10 seeded lung metastasis model, and B16F10 subcutaneous model. In all models, the Av3mEndo and the control Av3NullI vectors were administrated by tail vein injection. The blood level of endostatin was determined. Av3mEndo administration demonstrated systemic secretion of endostatin in much higher level than the endogenous endostatin of the controls, HBSS and Av3NullI treated mice. In colon liver metastasis model, systemic secretion of endostatin rendered extended survival in colon liver metastasis bearing mice. In B16F10 lung metastasis model, Av3mEndo vector demonstrated additional anti-metastasis effect over the control, Av3NullI. The results support the potential application of adenoviral vector-mediated endostatin secretion in anti-angiogenesis based cancer therapy.

Materials and Methods

Adenoviral vectors

Av3mEndo vector encoding secreted murine endostatin was constructed and characterized as described in report "Construction and characterization of a recombinant adenoviral vector encoding secreted murine endostatin" (1). The control vector, Av3NullI possess the same backbone gene except that no coding sequencing was in the expression cassette. Vectors used in this report were bulked up by tumor targeting group or Gene Therapy Core Technologies at GTI, which are listed below, Av3mEndo, Lot#TCA74A&B, 1.4 $x10^{11}$ particles/ml, particle/pfu ratio of 33.6; Av3mEndo, Lot#CTC7-98, 2.18 $x10^{12}$ particles/ml, particle/pfu ratio of 77.3; Av3NullI, Lot#TCA75, 2.4 x 10^{12} particles/ml, particle/pfu ratio of 21.8; and Av3NullI, Lot#TCA29B, 3.64 x 10^{12} particles/ml, particle/pfu ratio of 37.5.

ELISA detection of mEndo secretion

Secretion of murine endostatin was routinely determined by murine endostatin ELISA kit (CytImmune Sciences, Inc., Collage Park, MD) according to the manufacture procedure. Following the plate washed, the analysis was carried out in triplicate on 96-well ELISA plate with the unknown samples diluted by 4, 16, or 64-fold with final 50% of diluent 1 and 25% of diluent 2. Each 100-µl of mEndo standard and diluted unknown sample was dispensed into designated well. Following addition of biotin mEndo-conjugate and anti-mEndo antibody, the plates were incubated at room temperature for 3 hours. After the plate washed, the streptavidin-alkaline phosphatase was added. The plate was incubated at room temperature for 30 min followed by 20-min color development in the streptavidin-alkaline phosphatase reaction mixture. The absorbance was determined at 492 nm by ELISA reader (Bio-Rad). The standard curve was established and the concentration of the unknown samples was determined by extrapolation from murine endostatin standard curve.

Colon liver metastasis model

All the animal studies were designed by tumor targeting group at GTI and carried out at AntiCancer, Inc. under GTI's supervision. Male athymic CD-1 nude mice between 4 and 5 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). The mice were maintained in HEPA filtered environment with cages, food and bedding sterilized by autoclaving. Animal diets were purchased form Harlan Teklad (Madison, WI). Ampicillin 5% (v/v) (Sigma, St. Louise, MO) was added to the autoclaved drinking water. Mice were treated with Av3mEndo (Lot#TCA74A&B) or the control, Av3NullI (Lot#TCA75) vectors at 2×10^{11} particles/mouse by tail vein injection at final volume of 100 µl. The controls were also carried out with HBSS saline alone at 100 µl per mouse. Ten days post vector injection, the blood endostatin level was determined in all mice by murine endostatin ELISA kit (CytImmune, Collage Park, MD). Thirteen days post vector injection, poorly differentiated human stage IV colon adenocarcinoma (T3N1M1) was implanted by surgical orthotopic implantation to the top of the ascending colon approximately 1 cm away from the cecum after the serosa of the site has been striped. The intestine was then returned to the abdomen and the abdomen was closed in one layer with sterile 6-0 surgical sutures. After tumor implantation, there were 11, 17, and 16 mice survival in HBSS, Av3NullI, and Av3mEndo treated group, respectively. The animal survival was then monitoring through out the study. The %survival was calculated based on 100% survival of those mice survived after orthotopic surgical tumor implantation.

B16F10 lung metastasis model

Male C57BL6/J mice at the age between 8 to 9 weeks old were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice were treated with Av3mEndo (Lot#TCA74A&B), or the control, Av3NullI (Lot#TCA75) vectors at 2×10^{11} particles/mouse by tail vein injection at final volume of 100 µl. The controls were also carried out with HBSS saline alone at 100 µl per mouse. Two days post vector injection, lung metastasis was established by tail vein injection of B16F10 cells suspension at 5×10^{4} cells/mouse. Fourteen days post tumor cell injection, all mice were sacrificed and autopsied. Liver samples were collected for liver transduction analysis. Blood was collected for murine endostatin determination. Lung was collected for surface metastases determination by stereomicroscope.

Liver transduction analysis

Liver transduction was performed by Southern blot analysis following the standard protocols by GTI Gene Therapy Core Technologies. Genomic DNA was isolated from frozen liver samples of control, Av3NullI and Av3mEndo treated groups using the Qiagen Genomic DNA Isolation kit (). Frozen liver was minced and treated with protease at 55°C for 18 hours in the Hybaid oven. After centrifugation, DNA was then isolated from the supernatant using Qiagen column. DNA was digested with NcoI and resolved on 1% agrose-TAE. After transferred to a nylon membrane, the membrane was prehybridized in 5X Denhardts, 6X SSC, 10 mM EDTA, 0.5% SDS , and 0.1 mg/ml S. sperm DNA at 68 ± 2°C for 2 hours. The membrane was then hybridized with a 554 bp [^{32}p]-labeled sig-mEndo internal probe at 68 ± 2°C and washed in SSC/SDS containing buffers at 68 ± 2°C following the standard protocol. The sig-mEndo

internal probe was prepared from pAvmEndoLxr digested by XbaI and StuI at 37°C for 4 hours and gel purified as described (1). The standards of various # of copies of sig-mEndo chimeric DNA per hepartocyte were prepared with known amount pAvmEndoLx shuttle plasmid (1) added to 10 μg hepartocyte genomic DNA for quantitative analysis. The ^{32}p radioactivity of sig-mEndo chimeric DNA band was determined by Phosphor Imager analysis. The copy # of sig-mEndo chimeric DNA per hepatocyte was extrapolated from the standard curve generated as described above.

B16F10 subcutaneous model

Male C57BL6/J mice at the age between 11 to 12 weeks old were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice were treated with Av3mEndo (Lot#CTC7-98), or the control, Av3NulI (Lot#TCA29B) vectors at 6 x 10^{10} particles/mouse by tail vein injection at final volume of 100 μl. The controls were also carried out with HBSS saline alone at 100 μl per mouse. Two days post vector injection, B16F10 cells were implanted subcutaneously at 5 x 10^5 cells per mouse. Tumor volume was measured through out the study. Liver samples were collected for liver transduction analysis. Blood was collected for murine endostatin determination.

Tumor blood vessel immunostaining

Tumor blood vessel was identified by CD31 immunohistochemdistry in the frozen primary tumor samples. The immunohistochemistry was carried out by GTI Gene Therapy Core Technologies. Monoclonal antibody directed against mouse CD31 (PECAM-1, platelet endothelial cell adhesion molecule) was purchased from Pharmigen (San Diego, CA).

Results and Discussion

Naturally occurring colon liver metastasis model

To determine if systemic delivery of Av3mEndo vector can produce systemic secretion of murine endostatin above the endogenous level, male athymic CD-1 nude mice were treated with Av3mEndo vector at 2 x 10^{11} particles/mouse by tail vein injection. The controls were carried out with Av3NulI vector at identical dose or HBSS at equal volume. Blood level of murine endostatin was determined by ELISA on day 10 post vector injection (Fig 1A). As shown in Fig1B, mice treated with Av3mEndo vector demonstrated 5 to 8-fold higher levels of murine endostatin than the controls, Av3NulI or HBSS treated mice.

Since liver is probably the site with the highest and longest adenovrial vector transduction in body, we expected a higher local secretion of endostatin in liver than other sites. The systemic delivery Av3mEndo might bring more benefits toward liver metastasis reduction than metastasis in other sites. Therefore, we tested the strategy of systemic delivery of Av3mEndo in this naturally occurring metastasis model. Poorly differentiated human stage IV colon adenocarcinoma (T3N1M1) was implanted by surgical orthotopic implantation to the top of the ascending colon of vector treated on day 13 post vector injection (Fig 1A). Tumor implanted mice usually develop micro liver metastasis 7-10 days post orthotopic

transplantation with a few cases with lymphonode metastasis. Liver metastasis involves all liver lobes and metastatic lesions overcome all liver tissues, liver volume increases 3 to 10 times. Mice normally died of serious late stage liver metastasis. As shown in Fig1C, mice treated with Av3mEndo vector before tumor implantation demonstrated higher survival rate than the controls of Av3NullI and HBSS treated mice. Fifty three days post tumor implantation, there were 62.5% mice survived in Av3mEndo treated group, while there were only 11.8% and 18.1% mice survived in Av3NullI and HBSS treated groups, respectively. Although we can not distinguish prevention of tumor engraphtment or liver metastasis, results clearly demonstrated that secreted endostatin by Av3mEndo treatment benefited prolonged survival in this naturally occurring liver metastasis model.

<u>B16F10 melanoma lung metastasis model</u>

B16F10 lung metastasis model was established in C57Bl6/J mice by tail vein injection of 5 x 10^4 cells per mouse. Two days before tumor implantation, the mice were treated with Av3mEndo vector by tail vein injection at 2 x 10^{11} particles per mouse. Controls were carried out with either equal volume of HBSS or the equal amount of Av3NullI vector. Fourteen days post tumor implantation, blood was collected from all animals and analyzed by mEndo ELISA. As shown in Fig. 2A, the mice treated with Av3mEndo vectors all demonstrated higher level of murine endostatin at the average of 708 ± 435 ng/ml. In contrast, the control mice treated with HBSS and Av3NullI only showed the endogenous level of murine endostatin at the average of 50 ± 15 and 56 ± 19 ng/ml, respectively.

Fourteen days post tumor implantation, all mice were sacrificed and autopsied. Liver transduction was determined from Av3NullI and Av3mEndo treated mice by mEndo Southern Analysis. All Av3mEndo treated mice demonstrated Av3mEndo liver transduction with the average of 11 ± 4 copy number of mEndo DNA per hepatocyte with respect to 0 copy of mEndo DNA per hepatocyte in the Av3NullI treated mice.

The lung surface metastasis was determined from all mice. As shown in Fig. 2B, the control mice treated with HBSS showed the highest number of lung metastasis at the average of 109 ± 65 number of lung mets/mouse. Mice treated with Av3mEndo vectors demonstrated the reduction of lung metastasis to the average of 36 ± 18 number of lung metastasis/mouse (33% relative to HBSS control). However, mice treated with Av3NullI vectors also demonstrated the reduction of lung metastasis to the average of 42 ± 28 number of lung metastasis/mouse (39% relative HBSS control). This indicated that the majority of anti-metastasis effect (61%) was caused by Av3 backbone vector and some reduction (6%) was caused by murine endostatin secretion. Since the original in vivo efficacy study was done with i.p. injection of insoluble endostatin protein, the therapeutic level of endostatin is not clear. We can not exclude that higher blood level of endostatin might be needed for better efficacy. Designing better vector to secret higher level of endostatin is ongoing. The current study does prove that Av3mEndo expressed and secreted functional murine endostaitn as demonstrated in vitro. Systemic administration of Av3mEndo demonstrated sustained blood level of endostatin as demonstrated on day 16 post vector injection. The detailed in vivo kinetic study to determine the duration and level of expression is in planning. Results support that anti-angiogenesis

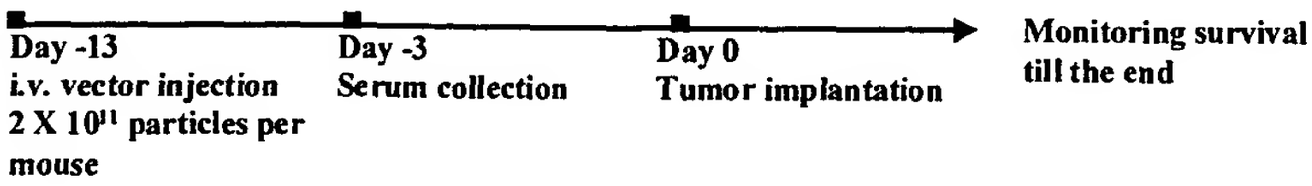
gene therapy of angiogenic inhibitor gene delivery might provide a potential mean to reduce lung metastasis.

B16F10 melanoma subcutaneous model

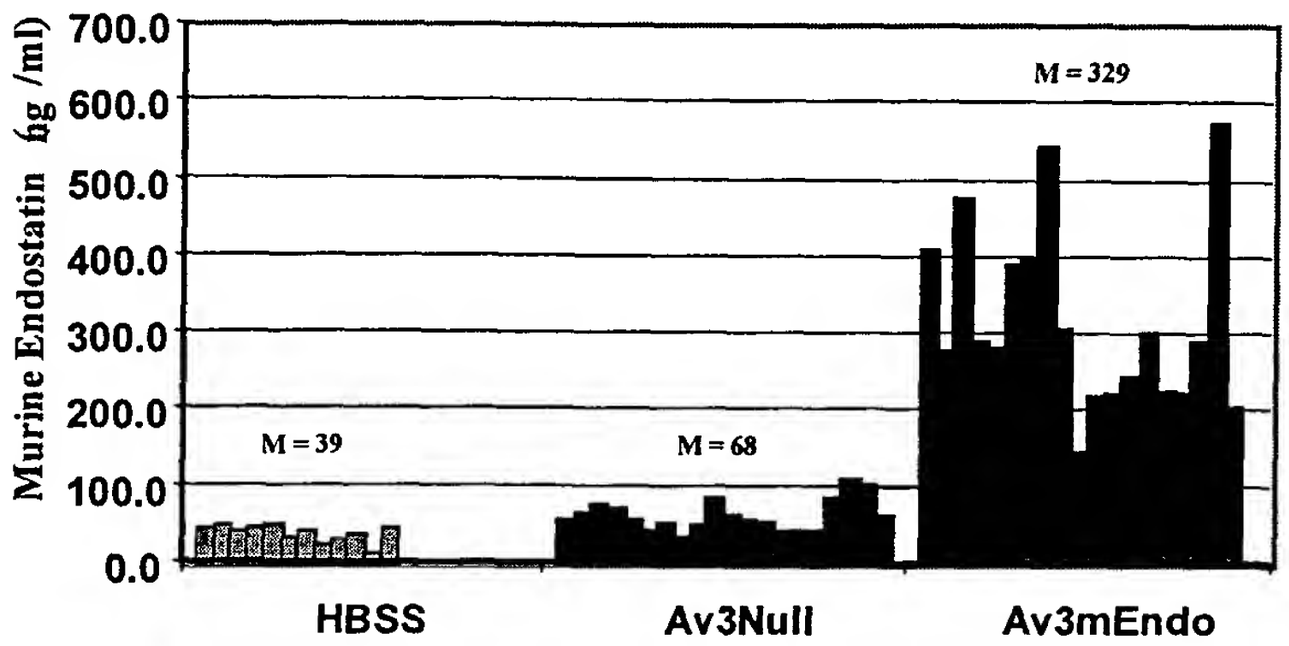
B16F10 subcutaneous model is one the models which demonstrated potent inhibition against tumor growth by systemic delivery of endostatin protein subcutaneously (2). If Av3mEndo vector can provide systemic secretion of endostatin at the therapeutic level, we should be able to reproduce the antitumor effect by systemic delivery of Av3mEndo vector. C57BLJ/6 mice were treated with Av3mEndo, or control Av3NullI vectors at 6×10^{10} particles/mouse, or equal volume of HBSS by tail vein injection. Two days post vector injection, subcutaneous tumor was implanted with 5×10^4 cells per mouse. Tumor volume was measured and recorded twice a week through out the study. As shown in Fig. 3B, Av3mEndo treated mice demonstrated the smallest tumor volume in average among the three treated groups. At the end of study, the blood level of murine endostatin was determined. As shown in Fig. 3C, all Av3mEndo treated mice demonstrated higher blood level of murine endostatin than the control mice. However, the level was not much above the level in the control mice except one mouse with blood level of endostatin at 500 ng/ml. The mouse with endostatin at 500 ng/ml showed the smallest tumor volume among all treated mice. All Av3mEndo treated mice demonstrated liver transduction by Av3mEndo at the average of 1.2 ± 0.7 copies per hepatocyte at the end of study. Since the blood endostatin was not much above the control, the anti-tumor effect was not dramatic.

Figure 1. Colon liver metastasis model: Systemic delivery of Av3mEndo demonstrated extended survival over the control, Av3Nul in the orthotopic colon cancer transplanted mice.

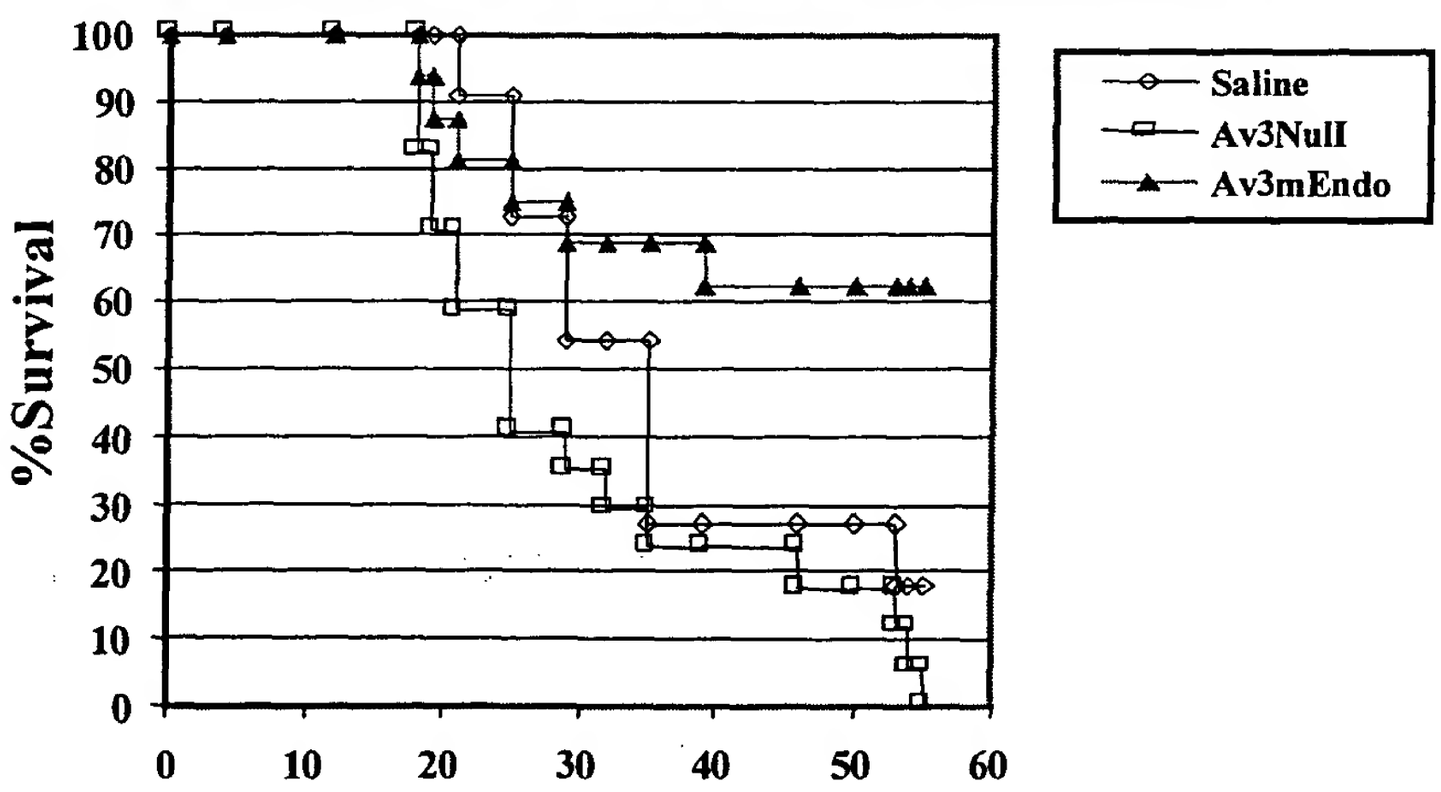
(A)



(B)



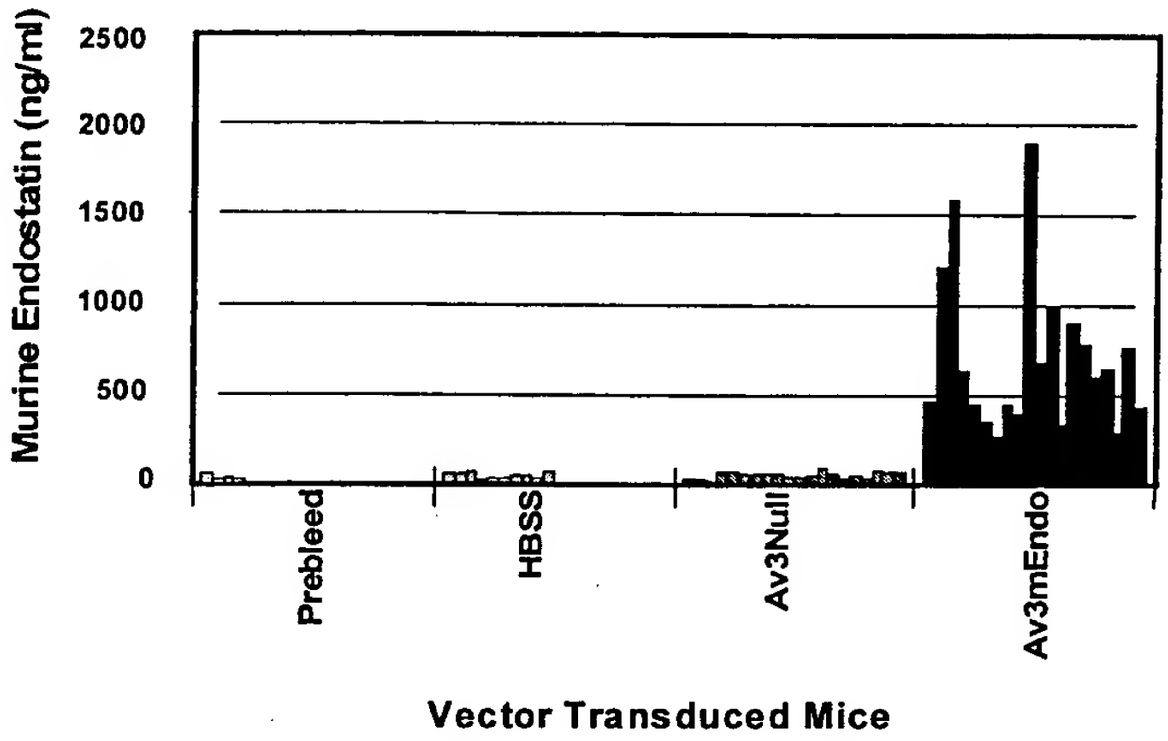
(C)



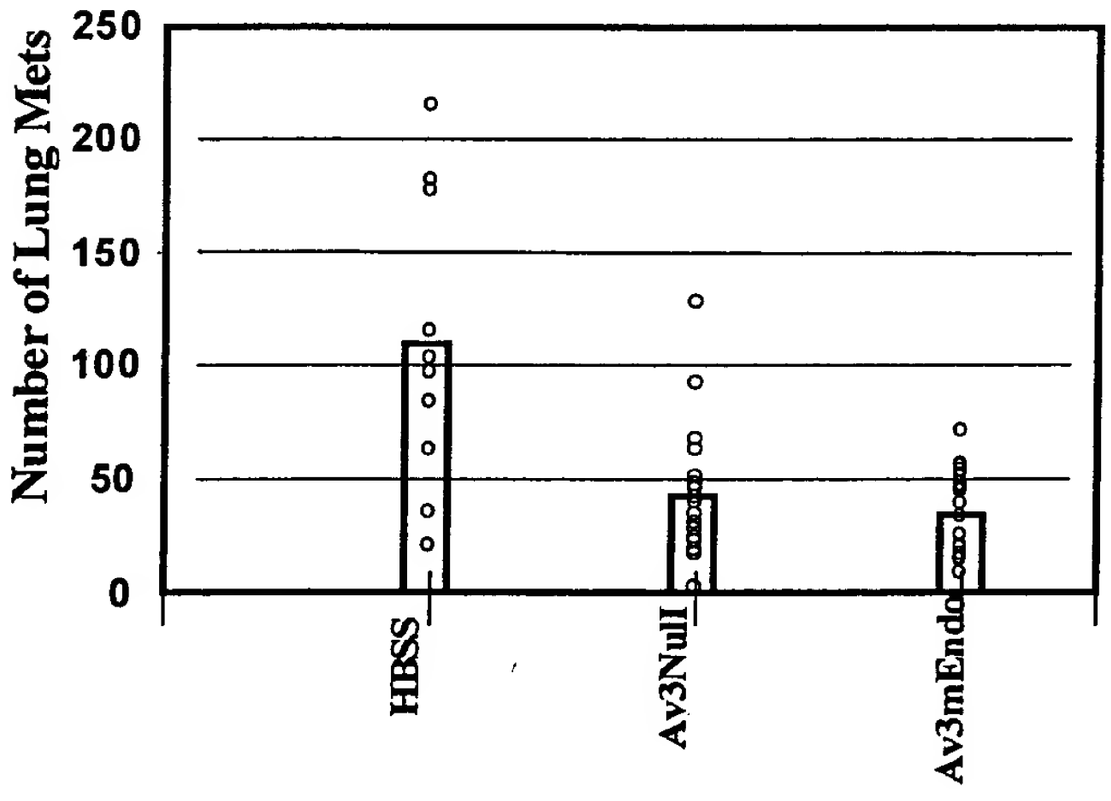
Athymic nude mice were treated with Av3mEndo, or control Av3NulI vectors at 2 x 10^{11} particle/mouse, or equal volume of HBSS by tail vein injection according to the schedule in panel (A). (B) The individual blood level of endostatin was determined by ELISA analysis as described in the text. The mean of endostatin level (M) in each group was labeled on the top of each group. (C) The survival was monitored through out the study based on 100% survival with the number of mice survived after tumor implantation. The number of animal (n) at 100% survival was listed in legends.

Figure 2: Mouse lung metastasis models: systemic delivery of Av3mEndo shows circulation of high level of murine endostatin at average of 708 ± 435 ng/ml 16 days post vector injection.

(A)



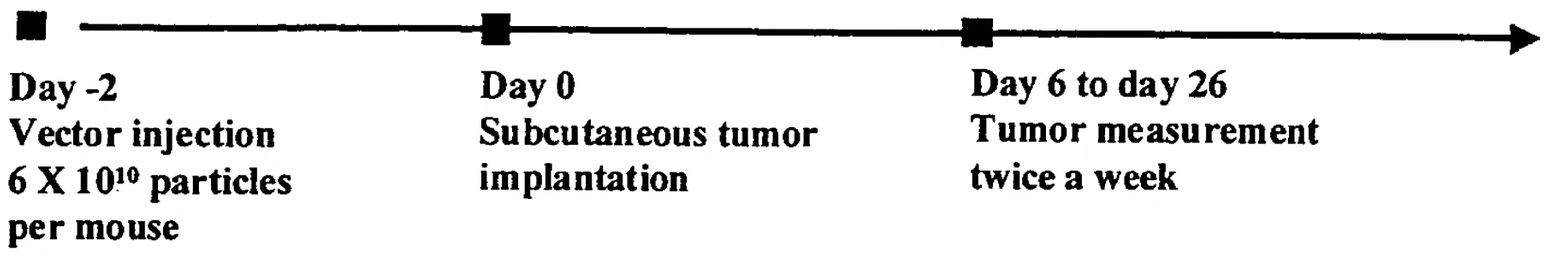
(B)



B16F10 mouse lung metastasis study was carried out as described in the text. C57BL/6J mice were treated with Av3mEndo (n=20, n represents number of animals per group), Av3Nul (n=20), and HBSS (n=12) by tail vein injection at 2 x 10^{11} particles per mouse. Two days after, lung metastasis was established by tail vein injection of B16F10 cells at 5 x 10^{4} cells per mouse. Fourteen days post tumor implantation, the study was ended. (A) Blood level of murine endostatin was determined at the end of study as described in the text. Each bar represents the blood level of mEndo from individual animal. (B) Surface lung metastasis was determined. Each open circle represents lung metastasis from individual animal. The bar represents the average lung metastasis of each group with different treatment.

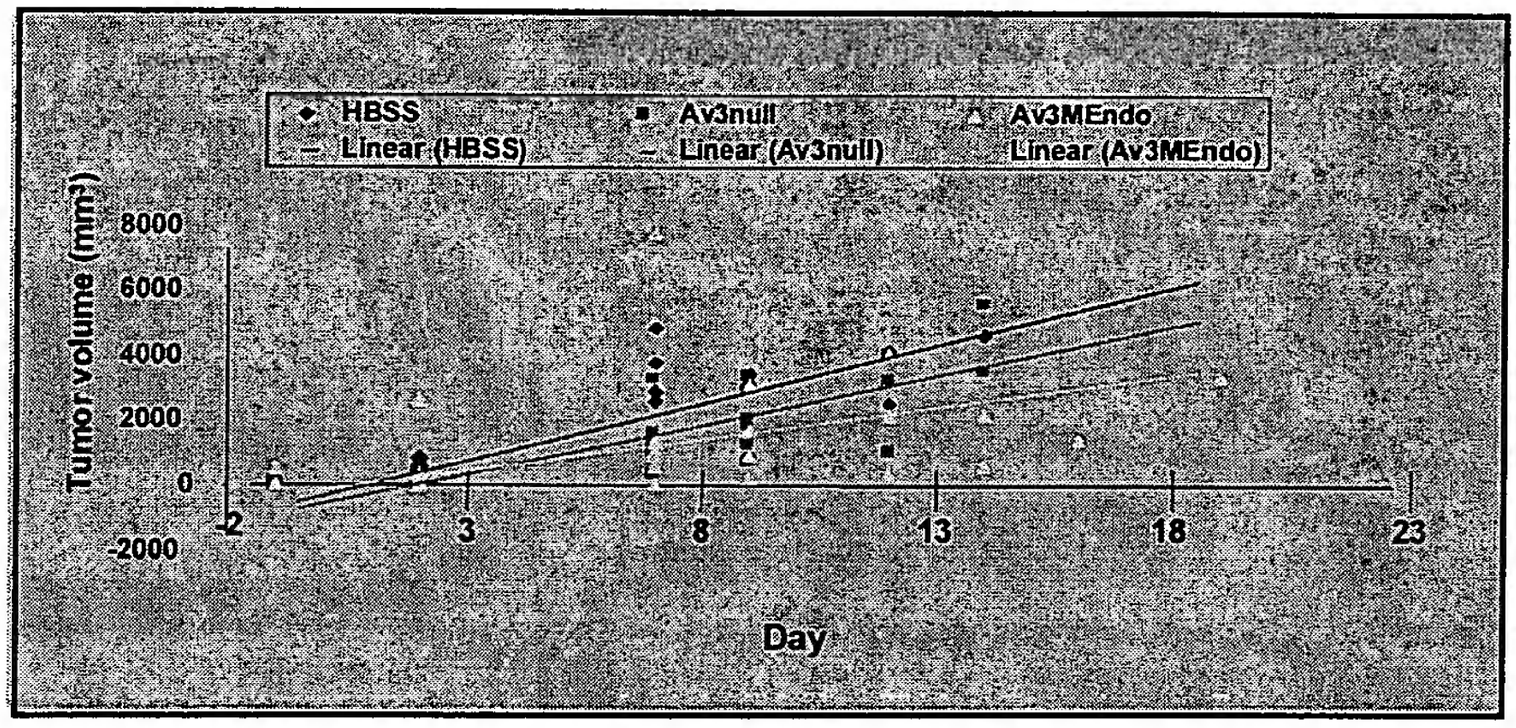
Figure 3. B16F10 subcutaneous model:

(A)

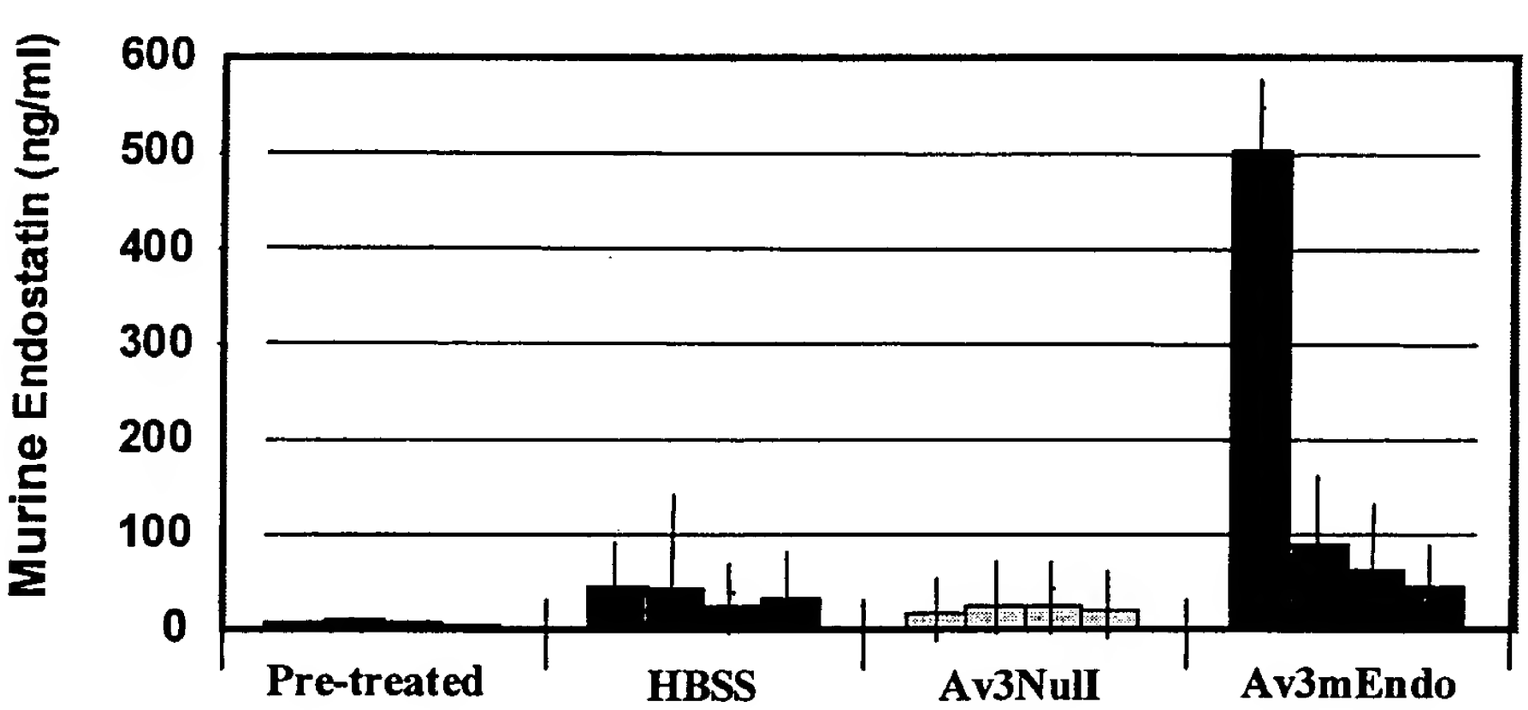


(B)

	# per group
HBSS	6
Av3Null	10
Av3mEndo	10



(C)



B16F10 mouse melanoma subcutaneous tumor model was carried out as described in the text. C57Bl6/J mice were treated with Av3mEndo (n=10, n represents number of animals per group), Av3Nul (n=10), and HBSS (n=6) by tail vein injection at 6 x 10^{10} particles per mouse. Two days after, B16F10 mouse melanoma cells were subcutaneouly implanted with 5 x 10^{5} cells per mouse. (A) Tumor volume was measured and recorded twice a week. Each symbol represents individual mouse. Line represents the average tumor volume of each group. (B) Blood level of murine endostatin was determined at the end of study as described in the text. Each bar represents the blood level of mEndo from individual animal.

References

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(2) O'Reilly, M.S., Boehm, T., Shing, Y., Fukai, N., Vasions, G., Lane, W.S., Flynn, E., Birkhead, J.R., Losen, B.R., and Folkman, J. (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88:277-285.